THE MOLECULAR WEIGHT OF ANTIBODIES*†

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Antibodies as modified serum globulins are of special significance in investigations on the nature of the serum proteins. Many of the relationships of antibodies to the normal serum globulins, however, have not as yet been thoroughly studied. Immunological studies (1–3) have indicated differences between antibodies formed in the horse and in the rabbit and a subsequent study in the ultracentrifuge by Heidelberger and Pedersen (4) revealed a striking difference in sedimentation constant and hence probably in molecular weight. The therapeutic superiority of rabbit antipneumococcus sera over horse sera (5) also makes further knowledge of the relationships among antibodies of various species highly desirable. Among these properties, molecular weight and the effect of pH changes on antibody activity as well as on molecular size are of especial interest.

In this investigation Types I, III, and VIII antipneumococcus antibody were produced in the horse, cow, pig, monkey, man, and rabbit and were purified by the dissociation methods described in (6, 7). The sedimentation constants (s) were measured using the Svedberg ultracentrifuge (8), and the diffusion constants (D) determined in the Lamm diffusion cell (9). From these values the molecular weight may be calculated from the formula given by Svedberg:

$$M = \frac{RTs}{D(1 - V\rho)}$$

and it is also possible to calculate the frictional ratio f/f_0 which gives an indication of the deviation from the compact spherical shape.

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† A preliminary note was published in *Science*, 1938, **87**, 372.

Data on the effect of protein concentration on sedimentation and a correlation of antibody activity and the ultracentrifugal pH stability range are also included.

Methods

For the study of ultracentrifugal sedimentation as well as diffusion the modified Lamm scale method (10) was used. A brief summary of the ultracentrifuge method has already appeared (4).

Sedimentation runs were made at varying speeds from 45,000 to 70,000 R.P.M., lower speeds being used for the faster sedimenting molecules. All sedimentation constants were corrected for density and viscosity (8).

Diffusion runs were made, taking photographs of the diffusing boundary at varying time intervals (9). All values represent the average of three exposures for each diffusion run and are corrected for the viscosity of the solution.

The partial specific volume (V) of a horse antibody preparation was determined as 0.715, using a micro balance and a 2 ml. pycnometer with a 1.9 per cent solution. This value was assumed to hold for all species showing the antibody to be a heavy molecule. For the other species the value 0.745, previously found for serum globulin was assumed to be valid.

Except as noted all experiments were made in 0.15 M sodium chloride solution (physiological saline) thus making it possible to determine sedimentation constant, diffusion constant, and percentage of antibody on the same solution. Total nitrogen was determined by the micro Kjehldahl method and antibody analyses for precipitin or agglutinin were carried out by the absolute methods (11, 12). The factor 6.32 was used to transform nitrogen content to total protein.

RESULTS

The process of purification was followed in several cases in the ultracentrifuge by runs on extracts prepared by washing the specific precipitate or agglutinate at 37° with physiological saline (7) and also on the 15 per cent salt extract before and after dialysis against 0.15 M salt. Table I shows the results for a horse, a pig, and a monkey antiserum. The pig antiserum was very weak and was first precipitated according to Felton (13, 6). Data on the relative amounts of antibody and of the heavy protein component are also given. It will be noted that no evidence of dissociation or change in the heavy component occurs in the horse or pig preparations at any stage in the purification.

By use of the new analytical separation cell (14), it was possible to show that all of the antibody in a Type I antipneumococcus horse

serum 902 I was contained in the heavy serum component. After centrifugation until optical observation indicated that all the heavy molecules had just passed below the partition into the lower compartment, it was found that no antibody could be detected in the solution from the upper chamber with either Type I pneumococci or specific polysaccharide. This was also found to be the case for a purified pig antibody solution 198 F described in (7) and containing 72 per cent antibody. The sedimentation diagram showed two components of $s = 18.7$ and 6.7. The heavy component, as determined from the sedimentation diagram, was present to the extent of 77 per cent, in excellent agreement with the value obtained by analysis for antibody, especially since the amount of heavy component is usually greater than the antibody content, *i.e.* not all heavy component is antibody (Table I).

Table II is a summary of sedimentation and diffusion constants, molecular weights, and frictional ratios for the different antibodies purified from various species. Total protein concentration and per cent of antibody in the solution are given. Since some variation of sedimentation constants with concentration has been found, s and D were determined at the same concentration. All of the preparations gave quite uniform sedimentation peaks. In two preparations from monkey Type III antipneumococcus sera[1] a definite amount of a heavy component was present in the 15 per cent salt extract. On dialysis to 0.15 M salt, in one instance it seemed to have precipitated along with the insoluble material which usually forms, since it was no longer present in the 8-58 dialyzed solution (Table I). Owing to the limited amount of available material, no separation of the two was possible. The human antibody solution[2] was prepared from the serum of a convalescent Type I pneumonia patient containing 0.15 mg. of agglutinin N per ml.

Two preparations were made by separation of the globulin fraction in the Tiselius electrophoresis apparatus (15); one from normal human

[1] Obtained in part through the courtesy of Dr. James D. Trask of the Department of Pediatrics, Yale University Medical School.

[2] Kindly furnished by Docent J. Waldenstrom, Upsala.

TABLE I

Sedimentation Constants of Horse, Pig, and Monkey Antibody at Various Stages in the Process of Purification

Species	Preparation No.	Description (7)	Concentration of solution	Antibody in solution (direct analysis)	Sedimentation constant $s_{20} \times 10^{13}$	Concentration of component in centrifuge	Appearance of components in sedimentation diagram
			per cent protein	*per cent*		*per cent protein*	
Horse Type I	902 I	Original serum	7.15	20.7			20 per cent heavy component in serum
	902 A	37° saline extract of washed specific precipitate	0.47	44	17.7	0.38	Single homogeneous component
	902 B	15 per cent salt extract at 37°	0.97	—	19.4*	0.95	" "
	902 B	Dialyzed against 0.15 M NaCl	1.35 0.58	57	16.7 17.2		" "
	902 C	$Ba(OH)_2$, $BaCl_2$ treated residue	0.74	66	16.5 4.0		Definite breakdown of molecule; two equal major components; inhomogeneous material
						per cent of total protein	
Pig Type I	W	Original serum diluted 1:10	0.84	1.6	17.7†	9.7	Homogeneous heavy component in 1:10 dilution of serum
	W_1A	Felton solution diluted 3:5	0.89	10.3	17.8	42.8	Both components homogeneous
					6.8	57.2	Light component probably normal globulin

* Correction for viscosity and density more uncertain.

† s of heavy component in serum.

TABLE I—*Concluded*

Species	Preparation No.	Description (7)	Concentration of solution	Antibody in solution (direct analysis)	Sedimentation constant $s_{20} \times 10^{13}$	Concentration of component in centrifuge	Appearance of components in sedimentation diagram
			per cent protein	*per cent*		*per cent of total protein*	
Pig Type I	W_1B	Washed specific agglutinate; extract with 15 per cent salt	0.50	—	18.5		Single homogeneous component
	W_1B	Dialyzed to 0.15 M NaCl	0.56	84	17.4		" "
	W_1C	$Ba(OH)_2$, $BaCl_2$ treated residue	0.63	83	19.1 2.7		Considerable breakdown converted largely into small components and inhomogeneous
Monkey Type III	8-58	Original serum	9.25	6.6			
		15 per cent salt extract	—	—	17.0 6.8	17.3 82.7	Both components homogeneous
		Dialyzed to 0.15 M NaCl	0.14	>27‡	7.2		

‡ Probably somewhat in the inhibition zone because of too large an excess of polysaccharide.

serum and the other 431-5 from a highly potent anti-egg albumin serum (Table II).

It was observed that the sedimentation diagrams of two rabbit $Ba(OH)_2$, $BaCl_2$ dissociated antibody preparations 456_2 B and 453 C (6, 7) seemed to be no different from that of the 15 per cent salt dissociated antibody. Two other preparations, C and F, from another serum 475 showed small amounts of several heavier, more inhomogeneous components. The bulk of the antibody was unchanged, however.

Table III shows the variation of sedimentation and diffusion constants with concentration for a horse antibody preparation 902 E,

TABLE II

Physicochemical Constants of Various Preparations

Species	Method of purification	Preparation No.	Total concentration of solution	Proportion of antibody	$s_{20} \times 10^{13}$	$D_{20} \times 10^{7}$	Calculated molecular weight	f/f_0
			per cent protein	*per cent*				
Pig	15 per cent salt extract*	W_1B	0.58	84	18.0	1.64	930,000	2.0
Cow (7)	" " *	D_2	0.64	100	18.1	1.69	910,000	2.0
Horse	" " *	902 E	0.22	49	19.3	1.80	920,000	2.0
Monkey	" " *		0.31	40	6.7	4.06	157,000	1.5
	" " *	8-58*†	0.14	>27	7.2	3.50	196,000	1.6
Human being	" " *		0.39	44	7.4	3.60	195,000	1.5
	(γ globulin from electrophoresis)		(0.63)	Normal globulin	(7.1)	(3.84)	(177,000)	1.5
Rabbit (7)	15 per cent salt extract*	456_2 A	0.19	86	7.0	4.23	157,000	1.4
	$Ba(OH)_2$, $BaCl_2$*	456_2 B	1.08	94	6.3	3.77	156,000	1.6
	$Ba(OH)_2$, $BaCl_2$*	453 C	0.83	89	6.3	3.80‡	158,000	1.6
	γ globulin by electrophoresis	431-5§	0.53	76	6.5	3.75	165,000	1.6

* Of Pn-anti-Pn specific precipitate or agglutinated pneumococci.

† Values subject to a larger experimental error because of extremely small amount of material.

‡ Some slight drift in D for various exposures. Mean value taken.

§ Anti-egg albumin.

TABLE III

Variation of Sedimentation and Diffusion Constant with Concentration of Solution for Horse Antibody 902 E

Concentration, *per cent protein*	0.22	0.45	0.90	1.35	1.80
$s_{20} \times 10^{13}$	19.3	18.0	16.7	15.4	13.7
$D_{20} \times 10^{7}$	1.80	1.83	1.84	1.63*	1.62

* 902 B.

made from the same bleeding of 902 I as preparation 902 B in Table I. For the study of concentration dependence and pH stability, the antibody from 200 ml. of serum was purified by salt dissociation of agglutinated pneumococci as in (7). Fig. 1 is a graph of the variation of s with concentration. The line is drawn through the points in Table III for 902 E and the other points were obtained with other horse, pig, and cow preparations at the concentrations indicated. It will be noted that the diffusion constant remains fairly constant, but shows some drop in more concentrated solutions.

Fig. 2 shows the variation of the sedimentation constant with concentration for the smaller antibody molecule. The data are taken from Table II. It will be observed that the rabbit and monkey solutions give a smooth curve over the range of concentration studied. The human antibody preparation, as well as the normal human γ globulin, seems to have a significantly higher sedimentation constant. Rabbit solution 456_2 B, however, run 5 months later at a concentration of 0.43 per cent seemed to have changed, giving a slightly higher value of $s = 7.0$.

Table IV is a tabulation of the effect of pH on the sedimentation constant and antibody activity for horse antibody solution 902 E. 1.5 ml. of 902 E 1.8 per cent solution in 0.15 M NaCl were mixed with 1.5 ml. of buffer solution made up in 0.15 M NaCl. The solutions were ultracentrifuged 10 minutes after mixing and also after standing 48 or 72 hours in the ice box. Antibody activity was determined by neutralizing and dialyzing against 0.15 M NaCl, after which samples were analyzed for agglutinin N and total N.

Antibody solutions 902 E and W1B were treated with 0.5 per cent phenol and let stand for 17 days. After this time no change could be observed in the shape of the sedimentation diagram and values of s of 16.4 and 17.9 $\times 10^{-13}$ were obtained in 0.9 and 0.23 per cent solutions for 902 E and W1B respectively.

Table V is a summary of the horse antibody preparations showing more than one component. In two instances antibody isolated from sera obtained early in the course of immunization contained only a single homogeneous component, while that from a bleeding after a longer period of immunization was markedly inhomogeneous. The wide spreading of peaks and inhomogeneity makes the sedimentation

constants only approximate in these instances. Rough estimates are also given for the total percentage of inhomogeneous material.

Antibody solution 902 K, prepared from the second bleeding of horse 902, and containing 26 per cent inhomogeneous material was run repeatedly in the separation cell, centrifuging the heavy component into the lower compartment until enough of the lighter components were available for analysis. This solution containing the lighter inhomogeneous components showed immediate agglutination with Type I pneumococci and 19 per cent of the total protein was antibody.

DISCUSSION

The present study is concerned chiefly with the ultracentrifugal behavior of antibody produced in various species of animals and purified by methods previously described (6, 7). To detect any alteration in molecular size during the purification, solutions were run in the ultracentrifuge at each step in the procedure. It will be noted (Table I) that all of the protein of low sedimentation constant can be washed away with saline at 0°, since solution 902 A, a saline extract at 37°, made after washing the specific precipitate at 0° until only traces of heat coagulable protein were present in the supernatant, contains only a single component of $s = 17.7 \times 10^{-13}$. At no stage in the salt dissociation procedure was there any indication of alteration in molecular size of the material (Table I). In the case of antibodies recovered from horse and pig sera the barium hydroxide dissociated material, however, showed definite breakdown in the ultracentrifuge although the antibody activity did not seem to be affected. The rabbit antibody seemed more stable to the barium treatment.

Using the salt dissociation methods (6, 7), it has been observed that preparations from different antisera give antibody solutions which differ very markedly in antibody content, and also that a preliminary extraction of the washed specific precipitate with 0.9 per cent saline at 37° yields a lower grade antibody than a subsequent 15 per cent salt extraction. Since in the original serum (902 I) the percentage of heavy component and of antibody is the same, and since the two salt extracts are indistinguishable in the ultracentrifuge, it is possible that some change affecting the antibody activity but not the molecular size might occur in purification. Thus it has already been

observed (16) that purified antibody solutions contain more agglutinin than precipitin although they correspond in the original sera. Moreover, in the case of pig serum W, Table I, the amount of antibody in the original serum was much lower than the amount of heavy component, yet a solution containing 83 per cent antibody was finally obtained, indicating that the other non-specific heavy component did not interfere with the purification.

The data in Table II indicate quite definitely that the purified antibodies from the various animal species fall into two groups, those in which the antibody has about the same molecular weight as the ordinary serum globulins and those in which the antibody seems to be a much larger molecule. Goodner and Horsfall (3) have also observed a distribution of antibodies in two groups with respect to complement fixing ability and lipid composition, *i.e.*:

Complement fixing: Rabbit, rat; guinea pig, sheep.

Non-complement fixing: Horse, man, dog; mouse, cat, goat.

These properties do not, however, correlate well with the molecular weight data, since human antibody (a small molecule) does not fix complement and a preparation of cow antibody (a large molecule)[3] showed definite complement fixation.

Since both monkey preparations showed definite amounts of a heavy molecular species, it is possible that both types of antibody are formed in the monkey.

It is also of considerable interest to observe that the frictional ratio f/f_0 calculated in the last column of Table II is large for both groups of antibodies, indicating that they are either not compact or not spherical, since f/f_0 is equal to one only for a spherical unhydrated molecule. The heavy antibody molecule has one of the highest frictional ratios yet observed in a protein. It is therefore not surprising to find a marked dependence of sedimentation constant on concentration (Table III and Fig. 1). The cow and pig values obtained are in good agreement with the horse values. It seems, therefore, advisable to take the extrapolated value of sedimentation constant to zero protein concentration of 19.8. With 1.71 as an average diffusion constant for the three species and 0.715 for the partial specific volume,

[3] Kindly tested by Dr. A. J. Weil of Lederle Laboratories.

990,000 would seem the best value for the molecular weight of cow, horse, and pig antibody, using the Svedberg formula given above. In the horse antibody solutions there was no evidence of dissociation in dilute solutions as has been found for thyroglobulin (17).

Horse antibody 902 E showed very definite streaming double refraction in less than 0.1 per cent solution and measurements[4] indicated a

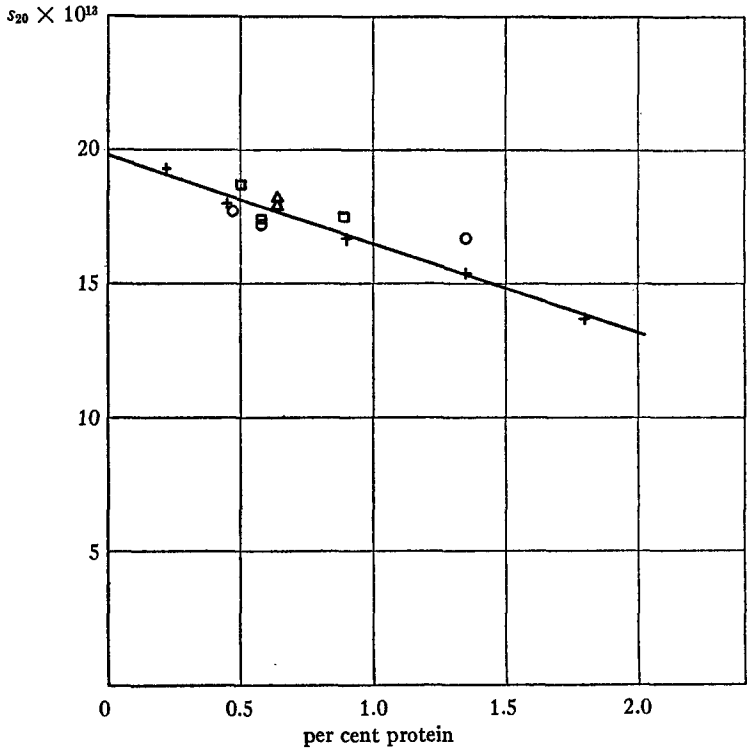

FIG. 1

+, horse 902 E. ○, horse 902 A and B. □, pig. △, cow.

length of not more than 100 mμ although the high dilution made accurate measurements very difficult. For a stiff cylindrical shaped molecule of molecular weight 990,000 and length 100 mμ, the ratio of the length to the diameter of the cylinder may be calculated as 25:1 as would be expected for a molecule with f/f_0 of 2.0.

[4] Kindly made by Dr. A. Snellman of this laboratory.

As shown in Table II and Fig. 2, the smaller antibody molecules also show definite variation of sedimentation constant with concentration. The diffusion constants also seem to show the same effect and the molecular weight in the rabbit and monkey preparations seems to be quite constant except for monkey 8-58 in which the weak solution available introduced a somewhat greater error. The best value for the molecular weight of rabbit and monkey antibody would be about 160,000, and since both the human antibody and the normal γ globulin both show a definitely higher sedimentation constant a probable average molecular weight would be about 185,000.

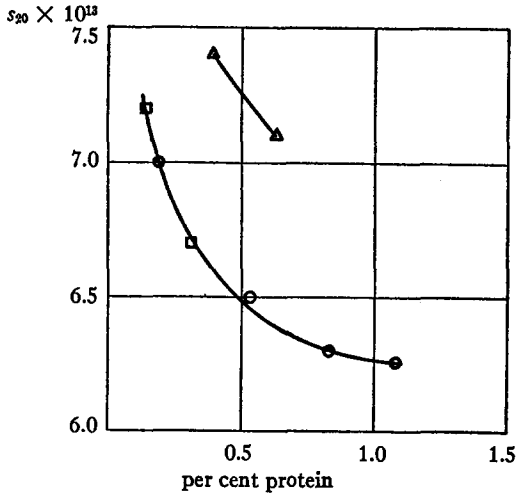

FIG. 2

○, rabbit. □, monkey. △, human being.

From the results in Table IV and Fig. 3, it will be seen that both antibody activity and molecular homogeneity are extremely stable to variation in pH, no appreciable change in activity occurring after 72 hours at pH 1.44 and after 48 hours at pH 10.9. The shape of the sedimentation diagram was unchanged between pH 3.4–10.9 although some association and dissociation seemed to take place below pH 4.88 and above pH 9.06 respectively. Some slight breakdown of the molecule was apparent at pH 1.44 with no loss of activity and complete loss of activity and destruction was found at pH 12.4. That this alkaline breakdown of the molecule may occur without loss of

TABLE IV

Effect of pH on Sedimentation Constant and Antibody Activity Horse Antibody 902 E. Concentration of Solutions in Centrifuge 0.9 Per Cent Protein

pH of solution	Total buffer in solution 0.15 M NaCl plus	Time of standing	Number of components in centrifuge	$s_{20} \times 10^{13}$ of main component	Antibody in solution	
					per cent	
1.44	0.015 M NaCl	10 min.	3	19.5		Also small amounts of components s = 6, 27
	0.05 M HCl	72 hrs.	3	20.2	52*	Small amounts of components s = 6, 30
3.41	NaAc 0.01 M	10 min.	1	18.2		Single symmetrical peak
	HAc 0.25 M	48 hrs.	1	17.6		" "
4.88	NaAc 0.025 M	10 min.	1	16.5		" "
	HAc 0.0125 M					
7.65	No buffer	10 min.	1	16.7	49	" "
9.06	$Na_2B_4O_7$ 0.01 M	10 min.	1	16.3		" "
10.9	Na_2HPO_4 0.025 M	10 min.	1	14.7	47†	" "
	Na_3PO_4 0.008 M	48 hrs.	1	15.3		" "
12.4	Na_2HPO_4 0.05 M	10 min.	2	6.7; 6.0		Two definite components complete breakdown
	NaOH 0.10 M	92 hrs.	2	4.5	0	Also some of a slightly lower component

* Antibody activity after 48 hours at pH 1.44. Neutralized solution showed main component s = 18.4 at 0.8 per cent concentration. Some slight breakdown of the molecule was still evident.

† Antibody activity after 72 hours in ice box, pH 10.9.

antibody activity may be seen from the data with the less drastic barium hydroxide dissociation methods (6, 7) as well as the barium hydroxide pig and horse preparations in Table I.

As noted by Heidelberger and Pedersen (4) not all preparations of horse antibody contained a single component. Even though the sera in the present experiments were free from preservative, many lots

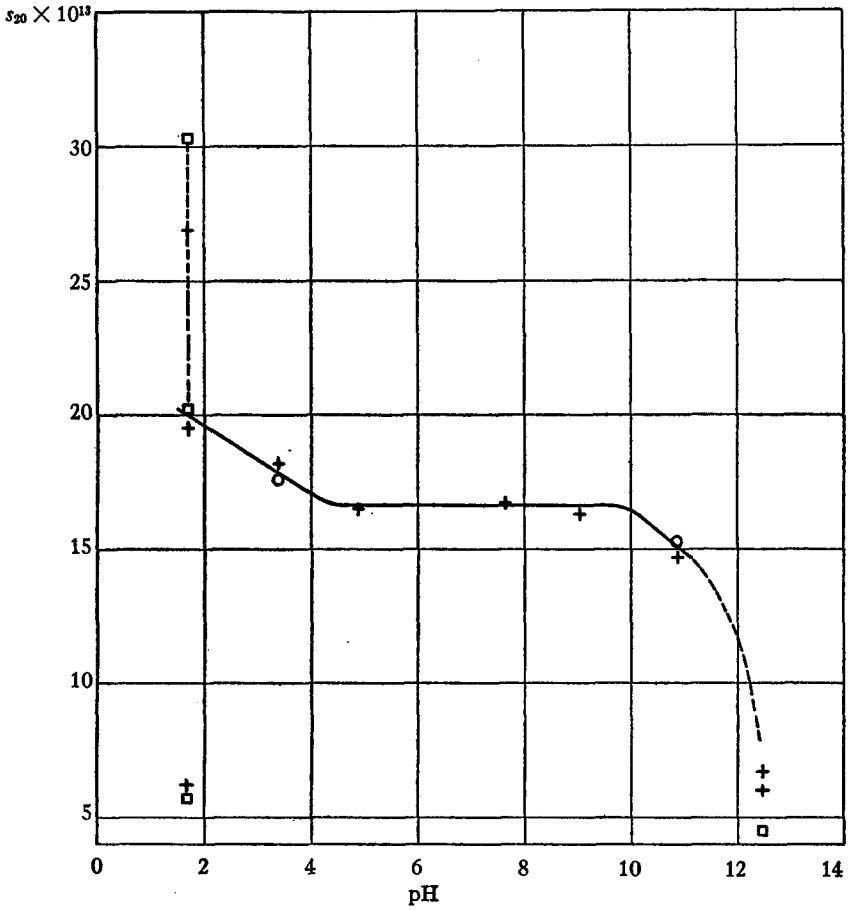

FIG. 3

+, 10 min. after mixing.
○, 48 hours " "
□, 72 " " "

showed inhomogeneous components in amounts up to 65 per cent of the total protein present. That this can occur without seriously impairing the antibody activity is apparent from the high degree of

antibody content of solutions 792 EE, 9093 B, and 909 E. A separation run in the analytical cell (14) also indicated that these degraded components possess antibody activity. Since in two of the horses 902 and Sn,[5] an early bleeding yielded a serum from which antibody solutions containing only a single homogeneous component could be prepared, and since in each of these instances, bleedings after longer

TABLE V

Horse Antibody Solutions Containing More than One Component

Antibody solution No.	Concentration of solution	Antibody content	s of various components		Degraded inhomogeneous material	Description
	per cent protein	*per cent*			*per cent*	
792 DD	0.10	37	20.1	8.6	53	
792 EE	0.5	96	19.0	5.6	58	Also some of an intermediate component
9093 A	0.05	57	19.7	7	65	
9093 B in 15 per cent NaCl			16.2	7.4		Two components
B	0.46	86	17.6	10	33	
909 E	0.18	97	18.4	9	40	Also some intermediate components
902 I B	0.58	57	17.2	—	0	Homogeneous 1st bleeding
902 K in 15 per cent salt			18.0	7.6	27	
902 K	0.67	49	18.0	12.0; 10; 4	26	Second bleeding after 1 yr. of continued immunization
Sn I	0.68	61	18.2	—	0	Data from (4)
II A	0.18	6.5	19.2	10; 8		Bleeding made several years later
B in 15 per cent salt			20.2	8		
B	0.63	30	18.6	17; 12; 7	22	

periods of immunization yielded solutions containing definite amounts of degraded material, it seems possible that the horse might develop means of breaking down the antibody into smaller aggregates without destruction of specific activity. This might be an indication of a mechanism for removal of an otherwise minor protein component

[5] Stockholm horse used in (4).

from the blood stream when the animal is artificially stimulated to produce large quantities of such a component. It is interesting to note that both the cow and pig preparations studied were from animals under immunization for a comparatively short time and that only homogeneous antibody solutions were obtained.

The relationship of the heavy component of horse serum to a normal heavy component frequently present in small amount in sera is of considerable interest. A preparation of horse globulin[6] which was thought to contain heavy component showed two components, one having the sedimentation constant of normal globulin and the other heavier. Both components showed fairly symmetrical peaks in the sedimentation diagrams. To a sample of this solution, some homogeneous horse antibody was added and the mixture ultracentrifuged. The presence of three components $s = 19.0$, 10.9, and 6.9×10^{-13} indicates the non-identity of the normal heavy components in this preparation with the antibody.

SUMMARY

1. Highly purified preparations of homogeneous antibody can be made by the salt dissociation methods (6, 7) without any change in sedimentation due to the method of purification.

2. Antibodies prepared from sera of various animal species fall into two groups as regards molecular weight; in one group cow, horse, and pig, a heavy molecule of molecular weight 990,000 is formed; in human being, rabbit, and monkey, the molecular size is that of the normal γ serum globulin. Both types of antibody molecules are either not compact or not spherical since the frictional ratios f/f_0 are 2.0 and 1.5 respectively.

3. Horse antibody shows an unchanged activity and sedimentation diagram between pH 3.44–9.06, although there is some aggregation at the more acid and some dissociation at the more alkaline pH. At pH 1.44 the antibody activity is unchanged but some breakdown of the molecule takes place. At pH 12.4 activity is destroyed and the molecule is completely broken down.

4. Some horse antibody preparations show evidence of breakdown

[6] Kindly supplied by Dr. F. E. Kendall.

of the antibody into inhomogeneous material on continued immunization over a long period.

We wish to thank Professor The Svedberg for his interest and for providing facilities for this work and Dr. Michael Heidelberger for supplying sera and making several of the antibody preparations especially for this purpose and for the interest taken in this work. We are also indebted to Dr. Kai O. Pedersen for many suggestions and continued guidance.

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